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Evaluation and optimization of lysis method for microbial DNA extraction from epiphytic phyllosphere samples

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ABSTRACT

Analysis of microbial communities in the epiphytic phyllosphere can be challenging, especially when applying sequencing-based techniques, owing to the interference of plant-derived biomolecules such as nucleic acids. A review of recent studies on the epiphytic microbiome revealed that both mechanical and enzymatic lysis methods are widely used. Here, we evaluated the effects of the two lysis methods on DNA extraction yield, purity, integrity, and microbial 16S rRNA gene copy number per ng of template genomic DNA under different extraction conditions. Furthermore, the effect on bacterial community composition, diversity, and reproducibility was examined using 16S rRNA gene amplicon sequencing. The enzymatic lysis method yielded one to two orders of magnitude more DNA, but the DNA quality was suboptimal. Conversely, the samples prepared using the mechanical method showed high DNA purity albeit lower yield. Unexpectedly, mechanical lysis showed a higher DNA integrity number (DIN) than enzymatic lysis. The 16S rRNA amplicon sequencing results demonstrated that the samples prepared via mechanical disruption exhibited reproducibly similar microbial community compositions regardless of the extraction conditions. In contrast, the enzymatic lysis method resulted in inconsistent taxonomic compositions under different extraction conditions. This study demonstrates that mechanical DNA disruption is more suitable for epiphytic phyllosphere samples than enzymatic disruption.

1. Introduction

The phyllosphere is the aerial region of the plant, which supports diverse groups of microorganisms that can live on the surface (epiphytic) and interior (endophytic) of plant tissues (Vorholt, 2012). The epiphytic phyllosphere is an important microbial habitat and plays a crucial role in the global carbon and nitrogen cycles (Bringel and Couée, 2015; Delmotte et al., 2009) as well as in plant health and growth (Wang et al., 2015). Epiphytic microorganisms are also vital in many industrial applications, such as *Pseudozyma* in bioplastic production (Sato et al., 2017; Watanabe et al., 2014) and lactic acid bacteria in the anaerobic forage conservation process (e.g., silage) (Holzer et al., 2003). Understanding the abundance, diversity, and functions of the epiphytic microbiome often depends on culture-independent molecular techniques (Lucaciu et al., 2019). Molecular analysis of the epiphytic microbiome necessitates applying additional pretreatment steps such as washing (Kadivar and Stapleton, 2003; Saito et al., 2007; Wang et al., 2019) and sonication (Cui et al., 2021; Yang and Crowley, 2000) to isolate and purify microbial cells. However, a cell pellet sometimes unavoidably contains residual plant debris, and DNA and PCR inhibitors released from the plant cells often interfere with downstream molecular analysis of the microbial communities (Dent et al., 2004; Ikeda et al., 2009; Saito et al., 2007). Particularly, chloroplast 16S rRNA genes may ultimately hinder the detection of the bacterial 16S rRNA gene in PCR amplification (Dent et al., 2004).

It is critical to develop a simple and reproducible DNA extraction method optimized for each sample type to explore the microbial community in environmental samples using a molecular approach. Typical DNA extraction protocols from an epiphytic phyllosphere sample comprise three major steps: pretreatment, cell lysis, and subsequent DNA purification. This study focuses on the second step, cell lysis, to minimize contamination from plant-derived biomolecules. Notably, cell lysis relies on mechanical or enzymatic disruption in most commercial DNA isolation kits. Mechanical disruption is typically based on bead-

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beating to break the cell membrane. The advantage of mechanical disruption is efficacy and concurrent sample homogenization; however, applications relying on intact chromosomes could be limited owing to DNA shearing (Bag et al., 2016; Corcoll et al., 2017; Pakpour et al., 2013; Salonen et al., 2010). Enzymatic disruption is more suitable for obtaining genomic DNA (gDNA) but may suffer from extraction bias owing to the limited spatial access to all target organisms and different sensitivity for different cell types (Robe et al., 2003; Saito et al., 2007). Using the Google Scholar database (accessed on 9/13/2022), we selected the first 100 recent articles published after 2020, searched using the keywords "phyllosphere" and "sequencing" and classified them based on the lysis method. A total of 70 studies employed commercial kits with mechanical disruption, whereas 30 used commercial kits with enzymatic disruption (data available upon request), indicating that both methods are widely used in different research areas. However, to the best of our knowledge, there is no study examining the effect of cell lysis methods and extraction conditions on DNA quantity and quality and subsequent molecular analysis of the phyllosphere microbiome.

Factors affecting DNA extraction efficiency by each lysis method include bead-beating time, speed, and temperature for mechanical disruption (Li et al., 2007) and temperature and contact time for enzymatic disruption (Qamar et al., 2017). For this study, we selected two commercially available DNA extraction kits employing different lysis methods and evaluated the effect of cell lysis methods under different extraction conditions on DNA extraction yield, purity, DNA integrity number (DIN), and bacterial 16S rRNA gene copy number per ng template gDNA as a measure of preferential lysis of bacterial cells over plant cells. Furthermore, the effect of DNA extraction methods on microbial community composition, diversity, and reproducibility was examined using 16S rRNA gene amplicon sequencing.

2. Material and methods

2.1. Sample and pretreatment

An epiphytic phyllosphere sample was collected from a 30-day-old alfalfa silage. Silage is a form of forage conservation in an anaerobic environment (Collins et al., 2017). Silage is typically based on fermentation and, thus, is a microorganism-rich phyllosphere niche. As a conventional pretreatment method for epiphytic phyllosphere samples to isolate and purify microbial cells minimizing plant material, each sample (5 g) was suspended in 45 mL of sterile 0.85% NaCl solution for 2 h at 20 °C, filtered through two cheesecloth layers, and centrifuged at 12,000 ×g at 4 °C for 15 min (Li et al., 2020; Saito et al., 2007; Wang et al., 2019). The supernatant was discarded, the pellet was stored at −80 °C until subsequent DNA extraction, and the solid residue was also collected and stored at −80 °C.

2.2. DNA extraction

Mechanical and enzymatic disruptions are two cell lysis methods commonly used for DNA extraction from phyllosphere samples. This study selected the DNeasy PowerSoil Pro kit and DNeasy Blood and Tissue kits from the same manufacturer (Qiagen, Hilden, Germany) to represent each lysis method. DNA extraction using the PowerSoil kit was conducted according to the manufacturer's instructions with the following modifications: (1) three different bead-beating times, i.e., 5, 10, and 15 min at (2) two temperatures, i.e., 20 °C and 30 °C. The protocol recommended by the manufacturer was bead-beating for 10 min at 20 °C. Mechanical cell disruption was performed using a Bead Mill 24 homogenizer (Fisher Scientific, Waltham, MA, USA) in a temperature-controlled room. DNA extraction using the Blood and Tissue kit was performed with the following modifications: (1) four incubation times with proteinase K (5, 10, 20, and 60 min) at (2) three temperatures (20 °C, 40 °C, and 56 °C). The manufacturer's protocol for prokaryotes was incubation for 10 min at 56 °C, and 30 min was recommended for gram-positive bacteria. A longer incubation time (60 min) was employed to evaluate the effect of lower incubation temperatures. The enzyme-sample mixture was incubated in a water bath set at different temperatures. The total DNA yield was obtained by repeated elutions until the DNA concentration was lower than 5 ng μL^{-1} (**Table S1**). All extractions were performed in triplicate. One gram of the solid residue on the cheesecloth was also subjected to DNA extraction using the DNeasy PowerSoil Pro kit to ensure the efficacy of the pretreatment process.

2.3. Determination of DNA yield, purity, and integrity

DNA yield and purity were determined using the Epoch 2 Microplate Spectrophotometer and TAKE3™ microvolume plate (Agilent Technologies, Santa Clara, CA, USA). An absorption ratio at 260/280 nm ($OD_{260/280}$) was used as an indicator of protein contamination, and the ratio of 260/230 nm ($OD_{260/230}$) was used for a secondary purity check. DIN was measured using the Agilent 2200 TapeStation system (Agilent Technologies, Santa Clara, CA, USA). Notably, DIN determines the level of DNA degradation by assessing the distribution of electrophoretic signals across the size range using a proprietary algorithm. The higher the DIN value, the better the integrity of the gDNA sample.

2.4. Relative abundance of microbial DNA in the DNA extract

Quantification of PCR (qPCR) targeting bacterial 16S rRNA gene was performed using the CFX Opus 96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA) in 20-μL reaction mixtures containing 10 μL of SsoAdvanced™ Universal SYBR Green Supermix (Bio-Rad, Hercules, CA, USA), 300 nM of each primer, and 2 μL of template DNA. A primer pair of 1055F and 1392R was used for amplification (Park et al., 2017). Standard curves for quantification were prepared in triplicate using double-stranded synthetic DNA fragments (gBlocks®, Integrated DNA Technologies, Coralville, IA, USA) as control templates. The primer sequences are presented in **Table S2**. The qPCR program was initiated at 95 °C for 10 min and continued at 40 cycles of 95 °C for 15 s and 59 °C for 1 min.

2.5. Amplificon sequencing and bioinformatic analysis

Bacterial 16S rRNA amplicon sequencing was conducted at GENEWIZ, Inc. (South Plainfield, NJ, USA). DNA samples were quantified using a Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA), and 30–50 ng DNA was used to generate amplicons using a MetaVx™ Library Preparation kit (GENEWIZ, Inc., South Plainfield, NJ, USA). The V3/V4/V5 hypervariable regions were analyzed using two amplicons—one each for the V3/V4 and V4/V5 regions. **Table S2** presents the primer sequences targeting each region. The amplicons were sequenced on the same run using the Illumina MiSeq™ with a 2 × 250 bp paired-end configuration. The Quantitative Insights Into Microbial Ecology (QIIME) 1.1.9, an open-source bioinformatics pipeline, was used for data analysis. Quality filtering on sequence reads was performed to remove low-quality or ambiguous reads. Beta diversity was calculated using weighted and unweighted Unifrac, and principal coordinate analysis (PCoA) was performed with R v.4.1.1 software. The sequencing data have been deposited at NCBI under accession numbers SRR22404852, SRR22404853, SRR22404854, SRR22404855, and SRR22404856.

2.6. Statistical analysis

One-way ANOVA analysis was employed with the Tukey Honestly Significant Difference test to assess differences between the extraction methods for DNA yield, purity, DIN, and normalized microbial 16S rRNA gene copy number. *P*-values were adjusted for multiple testing using the false discovery rate method. All statistical analyses were performed using the R v.4.1.1 software.

3. Results and discussion

3.1. DNA yield, purity, and integrity

The effect of the cell lysis methods on microbial DNA extraction from the phyllosphere sample was examined using two commercially available kits with modifications. The Blood and Tissue kit was used for enzymatic disruption, and the PowerSoil kit was used for mechanical disruption. As shown in Table 1, enzymatic disruption exhibited one to two orders of magnitude higher DNA yields (66.5–97.1 μg) than mechanical disruption (0.54–5.24 μg) regardless of the extraction conditions. However, such a high yield by enzymatic disruption was possible only after repeated elutions up to 16 times (**Table S1**). In contrast, mechanical disruption required a single elution (data not presented). In multiple elutions, similar amounts of DNA (6–20 μg) were retrieved for the first three to four elutions, and then the DNA concentrations gradually decreased.

At the first elution, the DNA yield was 10.39 ± 2.83 μg on average, only two times higher than the sample prepared via mechanical disruption at 20 °C (4.73 ± 0.41 μg). The $OD_{260/280}$ of the samples prepared by the enzymatic disruption ranged from 1.46 to 1.52 (Table 1), which is considered "appreciably lower" (<1.6) (Corcoll et al., 2017). Severe contamination by plant cell materials was observed with enzymatic disruption even in the mildest lysis conditions (i.e., incubation with proteinase for 5 min at 20 °C). Given that the recommended protocol by the manufacturer of the Blood and Tissue kit for the prokaryote is much more intense (i.e., incubation for 10 min at 56 °C for bacteria in general and 30 min at 56 °C for gram-positive bacteria), extra care should be taken when enzymatic disruption is used for microbial DNA extraction from an environmental sample containing plant materials. We performed an additional DNA purification step to improve the $OD_{260/280}$ using a commercial kit, but the purity was not noticeably improved (data not shown). The $OD_{260/280}$ for mechanical disruption was higher than 1.8, not exceeding 2.0 (Table 1), which is generally accepted as "pure" for DNA (Carrigg et al., 2007; Jorgez et al., 2006). For enzymatic disruption, as the lysis intensity increased, i.e., the incubation temperature or incubation time increased at fixed temperatures, the DNA yield decreased, but there were no significant differences in the $OD_{260/280}$. The highest DNA yield was observed in the mildest lysis condition (incubation for 5 min at 20 °C), indicating that further optimization of this method may result in a higher yield. The temperature significantly affected the DNA yield ($P < 0.001$) for mechanical disruption. DNA yields at 20 °C (4.73 ± 0.54 μg) were 10 times higher than those at 30 °C (0.57 ± 0.03 μg) regardless of the bead-beating time. At the same temperature, variations in the bead-beating time had no impact on the DNA yield and the $OD_{260/280}$. There were no significant differences in the $OD_{260/280}$ regardless of the extraction conditions. The $OD_{260/230}$ values were low (0.7–0.86) irrespective of the extraction methods, typically indicating the presence of polysaccharides from plant materials (Varma et al., 2007).

Higher DINs indicate larger DNA fragments, and low DINs indicate fragmented DNA. Sequencing prefers higher DINs. Unexpectedly, lower DINs (<2), i.e., more severe DNA shearing, were observed for enzymatic disruption regardless of the extraction conditions, whereas mechanical disruption samples showed significantly higher DINs (>5, $P < 0.001$). In general, mechanical disruption is the most popular and commercially available method with high throughput and high efficiency (Husseini et al., 2022), but it can result in extensive shearing of DNA. Enzymatic disruption is typically considered the most common alternative to mechanical disruption to extract longer DNA fragments. However, in this study, the DINs were consistently lower in the samples prepared with enzymatic disruption than those with mechanical disruption. Further optimization with enzymatic disruption, such as shorter incubation time and lower temperature, may improve DNA integrity. For mechanical disruption, as the bead-beating time increased, DIN decreased significantly at 20 °C ($P < 0.01$) and only numerically at 30 °C. This suggests that temperature is the controlling factor in minimizing DNA shearing for the mechanical lysis method.

3.2. Quantification of bacterial 16S rRNA gene

The total copy numbers of the bacterial 16S rRNA gene in the solid residue samples were two orders of magnitude smaller than the pellets (**Fig. S1**), suggesting that the pretreatment method used in this study was suitable for collecting bacterial biomass. The bacterial 16S rRNA gene copy numbers normalized by the template DNA concentration were used to assess the microbial DNA extraction efficiency from the epiphytic phyllosphere sample, where a lower value indicates high plant DNA content. Mechanical disruption exhibited 2–12 times higher bacterial 16S rRNA gene copies per ng template gDNA ($2.7 \pm 0.1 \times 10^7$–$6.5 \pm 0.1 \times 10^7$) than enzymatic disruption ($5.2 \pm 0.0 \times 10^6$–$1.8 \pm 0.0 \times 10^7$) (Fig. 1). The copy numbers were consistent for mechanical disruption when extraction was performed at the same temperature irrespective of the bead-beating time, suggesting that the extraction temperature is a controlling factor for the preferential lysis of bacterial rather than plant cells (Fig. 1b). At 30 °C, two times higher copy

Table 1
Effects of cell lysis methods and extraction conditions on DNA yield (μg), purity (absorbance ratios at 260/280 nm ($OD_{260/280}$) and 260/230 nm ($OD_{260/230}$)), and DNA integrity number (DIN). The numbers in the parentheses indicate the standard errors of the estimates.

Blood and Tissue kit

Incubation	Temperature	20 °C				40 °C				56 °C			
	Time (min)	5	10	20	60	5	10	20	60	5	10	20	60
DNA total yield		97.1 (4.6)	96.6 (5.4)	80.9 (7.3)	67.9 (1.0)	80.9 (1.8)	74.1 (0.5)	70.0 (0.2)	57.0 (6.2)	74.3 (3.2)	77.3 (10.4)	80.1 (3.3)	66.5 (2.7)
DIN		1.33 (0.29)	1.40 (0.1)	1.20 (0.16)	1.00 (0.00)	1.2 (0.28)	1.13 (0.12)	1.00 (0.00)	1.05 (0.05)	1.00 (0.00)	1.27 (0.05)	1.53 (0.12)	1.13 (0.09)
$OD_{260/230}$		0.73	0.73	0.74	0.73	0.73	0.73	0.73	0.72	0.70	0.70	0.71	0.71
$OD_{260/280}$		1.49	1.46	1.52	1.51	1.49	1.50	1.52	1.50	1.50	1.47	1.49	1.50

Power Soil kit

Incubation	Temperature	20 °C			30 °C		
	Time (min)	5	10	15	5	10	15
DNA total yield		4.2 (0.1)	4.8 (0.2)	5.2 (0.1)	0.5 (0.0)	0.6 (0.0)	0.6 (0.0)
DIN		6.23 (0.12)	5.83 (0.17)	5.37 (0.05)	6.33 (0.12)	6.20 (0.08)	6.07 (0.09)
$OD_{260/230}$		0.81	0.86	0.78	0.80	0.82	0.79
$OD_{260/280}$		1.85	1.84	1.85	1.80	1.98	1.85

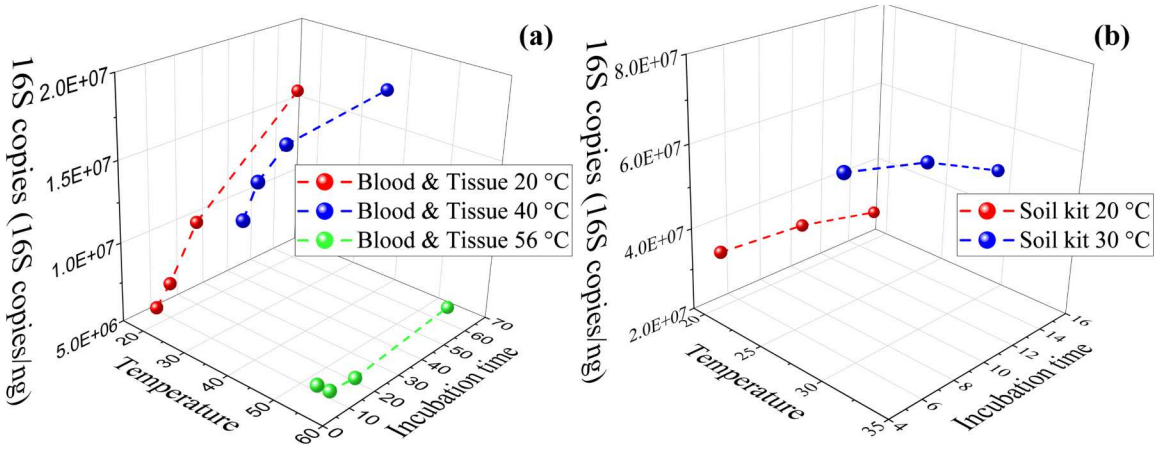

Fig. 1. Copy numbers of bacterial 16S rRNA gene normalized by the template DNA amount in the samples prepared using (a) enzymatic and (b) mechanical lysis methods under different extraction conditions.

numbers ($6.5 \pm 0.1 \times 10^7$) were detected than at 20 °C ($3.4 \pm 0.0 \times 10^7$). For enzymatic disruption, there were significant variations in the gene copy numbers depending on extraction conditions (Fig. 1a). At 56 °C, no significant differences were observed in the copy number as the incubation time varied. At 20 °C and 40 °C incubation temperatures, the 16S rRNA gene copy numbers increased as the incubation time increased. The results suggest that mechanical disruption is more suitable for the preferential lysis of bacterial cells than enzymatic disruption.

3.3. Composition and diversity of the bacterial community

Bacterial 16S rRNA amplicon sequencing was performed on selected DNA extracts in duplicate to examine the effects of different DNA extraction methods on the patterns of soil bacterial community structure and diversity. The sequences per sample ranged from 15,094 to 445,904, clustered at a 97% sequence identity threshold. A total of 2767 OTUs were identified at both phylum and genus levels. Depending on the extraction conditions, distinctive variations were observed in the bacterial community compositions between the samples prepared via enzymatic disruption. In contrast, the samples prepared via mechanical disruption exhibited consistent community compositions irrespective of the extraction conditions (Fig. 2). At the phylum level, there was a high abundance of unclassified phyla (72%) in the samples prepared via enzymatic disruption for 5 min at 20 °C (BT 5m20c) but not in the samples prepared using the same lysis method for 60 min at 40 °C (BT 60m40c, <5%) (Fig. 2a). In the samples prepared via mechanical disruption, the compositions were consistent. In all the samples, firmicutes was the predominant phylum, followed by proteobacteria, actinobacteria, and cyanobacteria. At the genus level, BT 5m20c exhibited the highest abundance of unclassified genera (up to 88%), whereas BT 60m40c displayed the lowest abundance of unclassified genera (20%). A positive correlation between small-sized (sheared) DNA and an abundance of unclassified sequences was previously reported (De Lipthay et al., 2004; Desneux and Pourcher, 2014; Teng et al., 2018). However, in this study, the DIN did not correspond to the abundance of unclassified sequences (Table 1). It can be speculated that the proportion of plant DNA may have been significant in the extracts prepared via enzymatic disruption, and the DINs did not represent the DIN of microbial DNA. Simultaneously, microbial DNA may have been released earlier than plant DNA and may have been exposed to the proteinase longer, resulting in more small-sized DNA in BT 60m40c than in BT 5m20c. The samples prepared using mechanical disruption also showed consistent community compositions at the genus level regardless of the extraction conditions. In all samples, *Pediococcus* was a dominant genus, followed by *Weissella*, *Lactobacillus*, and *Lactococcus* (Fig. 2b). Previous

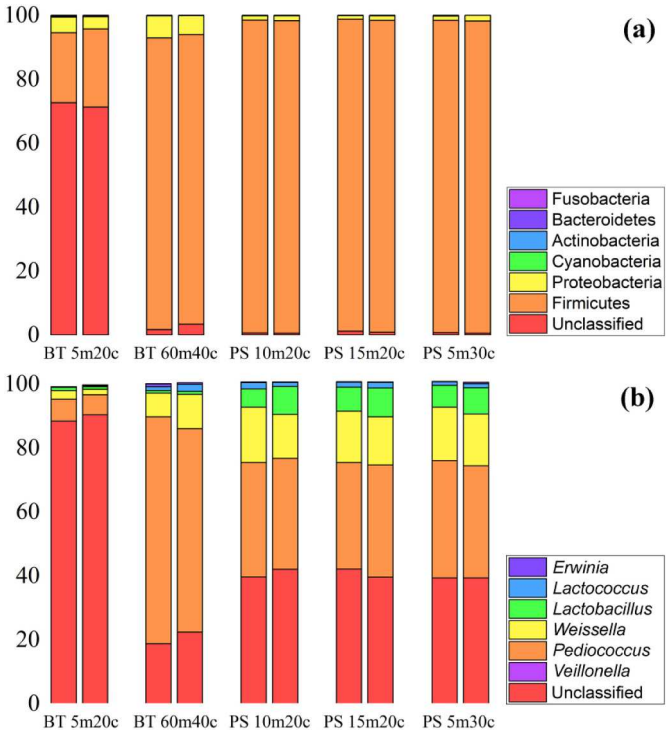

Fig. 2. Comparison of relative abundance of 16S rRNA OTUs in the samples prepared using two lysis methods under different extraction conditions at the (a) phylum and (b) genus levels. Each bar represents one replicate sample. BT and PS represent the samples prepared using the Blood and Tissue and PowerSoil kits, respectively. The numbers before m and c characters represent the incubation time and temperature, respectively.

studies have shown that differences in the bacterial cell wall and membrane structures cause DNA extraction to be more or less effective from some organisms. Such a discrepancy can introduce bias in the estimates of relative abundances of microbes in samples; thus, mechanical lysis methods are considered less biased than enzymatic lysis methods (Carrigg et al., 2007; Krsek and Wellington, 1999). Indeed, in this study, the samples prepared using the mechanical lysis method exhibited consistent microbial community compositions irrespective of the extraction conditions.

PCoA analysis also confirmed that mechanical disruption allowed consistent analysis of microbial community structure (Fig. 3). The first

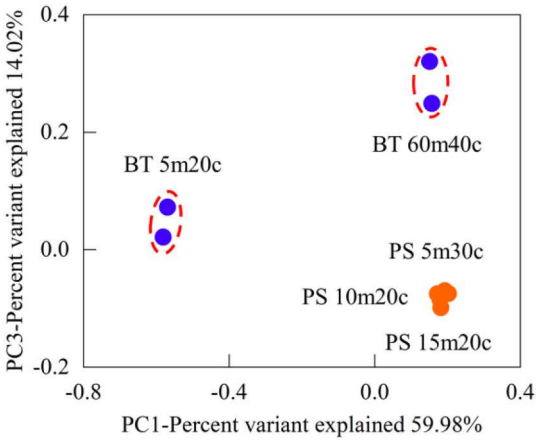

Fig. 3. PCoA plot of 16S rRNA OTU abundance in all samples. BT and PS represent the samples prepared using the Blood and Tissue and PowerSoil kits, respectively. The numbers before m and c characters represent the incubation time and temperature, respectively.

axis of the PCoA explains 59.98% of the variance, whereas the second axis explains 14.02% of the variance from the samples prepared using the Blood and Tissue and PowerSoil kits under different conditions. All duplicate samples were clustered close to each other, suggesting that both lysis methods are reproducible. Irrespective of the extraction conditions, the samples prepared via mechanical disruption were clustered together, whereas those prepared via enzymatic disruption were located separately in response to the different extraction conditions. This indicates that the mechanical lysis method is less biased than the enzymatic approach.

4. Conclusions

We hypothesized that an optimized cell lysis protocol would allow preferential bacterial cell lysis over plant cells. With enzymatic disruption, the DNA quality was suboptimal for downstream applications, and more importantly, the bias in the microbial community profiles was prominent, albeit with high DNA yield. In the meantime, the samples prepared via mechanical disruption exhibited high DNA quality and consistent microbial compositions irrespective of extraction conditions. Therefore, this study demonstrates that the mechanical disruption method is more suitable for epiphytic phyllosphere samples than the enzymatic disruption method, and disruption at 20 °C for 5 min was found to be optimal for the fermented forage samples used in this study.

Declaration of Competing Interest

None.

Data availability

The sequencing data was deposited at NCBI

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mimet.2023.106677.

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